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EFFECTS OF β-ECDYSONE ON MOLT-LINKED DIFFERENTIATION *IN VITRO*

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The occurrence of morphogenic activity in cultured tissues and organs of insects has been reported by a number of researchers (see Marks, 1970). However, the results of these studies have not always been easy to interpret. In their work with *Drosophila,* both Demal (1961) and Schneider (1966) pointed out that the stage of development of the tissue donor greatly affected the amount of morphogenesis that occurred, and Mandaron (1971) showed that α-ecdysone induced differentiation in explanted imaginal disks. Further, Oberlander and Fulco (1967) and Oberlander (1969) showed that the presence of ecdysone analogs in the culture medium enhanced morphogenic development in wing imaginal disks of *Galleria.*

Regenerate appendages in cockroaches may be formed during any instar, including those that occur within the egg (Bullière, Bullière, and Sengel, 1969). In nymphal cockroaches, a regenerate complete with setae and spines can be formed within a single instar only if the leg is removed early in the instar. If the leg is removed late in the instar, only an undifferentiated papilla is formed (O'Farrell and Stock, 1953).

Marks and Reinecke (1965) showed that incubation of very young leg regenerates of cockroaches with prothoracic glands from older insects resulted in a loss of organotypic activity and atrophy of the migrating cells. Since then, Bullière and Bullière (1970) convincingly demonstrated that the initiation of leg regeneration can be inhibited by treatment with molting hormone *in vitro.*

Marks and Leopold (1971) showed that treatment of mature (25-day-old) leg regenerates with β-ecdysone induced differentiation of tormogen and trichogen cells from the epidermis and that the frequency with which seta formation occurred was related to the age *in vivo* of the explant. Thus, *in vitro* the same dose of ecdysone that inhibits the initiation of regeneration enhances the development of a mature regenerate. Clearly, then, the presence of molting hormone, either exogenous or endogenous, has a pronounced effect on the course of development in cultured tissues and organs, and the effect is dependent on the age *in vivo* of the target tissue.

A second process that is related to development and can be initiated by the presence of molting hormone *in vitro* is cuticle deposition (Agui, Yagi and Fukaya, 1969; Marks and Leopold, 1970; Oberlander and Tomblin, 1972). The deposition of cuticle by cockroach leg regenerates *in vitro* also depends to some extent on the age of the tissue *in vivo* (Marks and Leopold, 1971), but far more so on the amount of exogenous molting hormone and the duration of exposure to it (Marks, 1972a).

Thus, morphogenesis in cockroach leg regeneration depends on the age *in vivo* of the explant and is linked with the process of cuticle deposition and molting. To

see whether we could define more precisely the relationship between these two processes, we undertook an *in vitro* study of the effects of the molting hormone β-ecdysone on seta formation and cuticle deposition in leg regenerates of cockroaches of different ages.

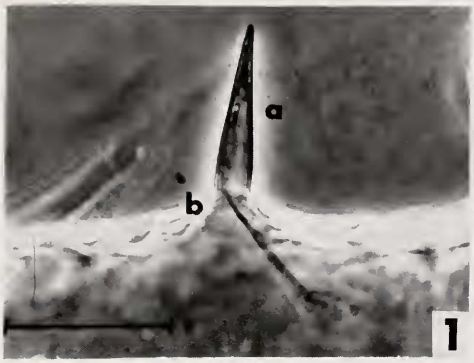
Methods

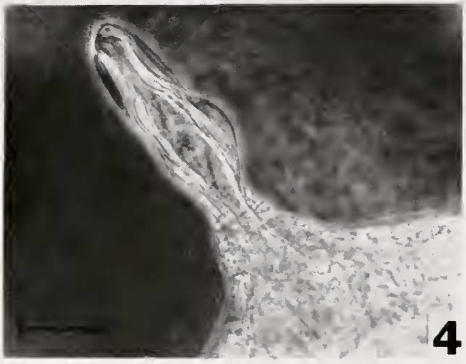
Newly molted late-instar nymphs of the cockroach, *Leucophaea maderae* (F.), were removed from the colony and held for 24 hr. Then the mesothoracic legs were removed at the coxo-trochanteral joint, and the insects were held an additional 10–30 days in paper cups and fed dog chow and water while leg regenerates developed. The insects were then surface-sterilized, and the leg regenerates were dissected from the coxal stump. The explants were placed under dialysis strips in multipurpose tissue chambers (Rose, 1954), and the chambers were filled with M20 culture medium (2 ml) supplemented with 7.5% fetal calf serum (fcs) (Marks, 1973). Crystalline β-ecdysone dissolved in water (1 μg/μl) was injected into the chambers with a microsyringe. In the first set of experiments, doses of 1, 5 and 10 μg/ml were given 24 hr after explanation and allowed to remain in the chambers for 5 days. In a second set of experiments, β-ecdysone (2.5 μg/ml) was injected into the chambers along with puromycin (0.5 μg/ml) and allowed to remain for 3 days. After exposure, all chambers were rinsed twice, refilled with culture medium and incubated at 27° C. The explants were examined by phase contrast microscopy after 10 days of incubation and again after 14 days. The criteria for the presence of cuticle were those of Marks (1972a), and each specimen was examined carefully for identifiable setae. Since many kinds of aberrant surface sculpture appeared on the explants when the cuticle was deposited, a structure was scored as a seta only if the trichogen cell or the shaft of the seta could be identified within the tormogen cell or socket. The outline of the explant was scanned for setae in profile (Fig. 1); if none was found, a detailed search of the surface of the explant was made at higher power (Fig. 2). The frequencies of occurrence of seta formation and cuticle deposition were measured for a minimum of 10 specimens for each dosage. The data were transformed—frequencies to probits and dosages to logs—to straighten the otherwise sigmoid curve, and a standard regression analysis was run to permit extrapolation.

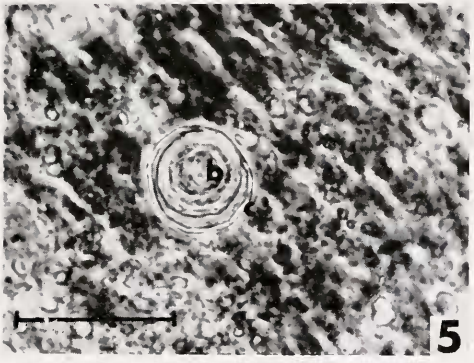
Results

Numerous types of sculpture were found on the cuticular surface of treated explants; these included fixed spines, pegs, unidentified ring-shaped ridges that may have represented aberrant tormogen cells, and scale-like sculpture (Figs. 3–6). Many of the pits and fixed spines occurred regularly when older specimens were given large doses of the hormones, but other structures (for example, the scale-like sculpture) were observed occasionally and then only on small portions of the explant.

In young tissues up to 20 days *in vivo*, as many as 40% did not respond to molting hormone, perhaps because of damage during explantation. To eliminate these unresponsive explants from consideration, we divided the frequency of seta formation by the frequency of cuticle deposition to obtain a ratio of seta to







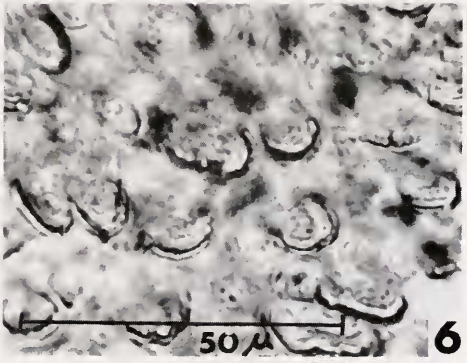


FIGURE 1. Seta shown in profile. Shaft (trichogen *a*) is seated in the socket (tormogen *b*); 26 days *in vivo;* 24 days *in vitro.*

FIGURE 2. Seta shown in surface view. Sclerotization of tormogen (a) and trichogen (b) is incomplete; 26 days *in vivo;* 24 days *in vitro.*

FIGURE 3. Multicellular spine formed on surface of explant is well-sclerotized; 26 days *in vivo;* 24 days *in vitro.*

FIGURE 4. Cuticular peg is of multicellular origin; 24 days *in vivo;* 30 days *in vitro.*

FIGURE 5. Circular pit contains ring-shaped cell (b), which may represent aberrant tormogen cell, within a circular ridge of cuticle (c); 26 days *in vivo;* 28 days *in vitro.*

FIGURE 6. Noncellular, scale-like, cuticular processes appear in patches and resemble sculpture found on mature legs; 26 days *in vivo;* 22 days *in vitro.*

cuticle. This ratio provided us with a measure of the frequency with which tissues that produced cuticle also produced setae (Table I).

In the 10- and 15-day age groups there were no significant differences in the frequency of seta formation or of cuticle deposition among either dose levels or age groups. The mean frequency for cuticle deposition was 61.2% and for seta formation, it was 5.7%. The resultant seta cuticle ratio varied from 0 to 12. The difference in frequency between the 15- and 20-day age groups is significant at the 95% level of confidence for both cuticle deposition and seta formation. The frequency of cuticle deposition was approximately 95% for all dose levels for all

TABLE I

The effect of age of explant in vivo *and dose of β-ecdysone on cuticle deposition and seta formation by cockroach leg regenerates* in vitro

Age (days) of explant *in vivo*	Dose* (5-day exposure)		Number specimens	Percentage of response of		
	μg/ml	μgd		Cuticle	Seta	Seta/cuticle
10	1	5	14	57.1	0	0
	5	25	17	47.0	5.9	12
	10	50	14	64.3	7.1	11
15	1	5	15	66.7	0	0
	5	25	15	60.0	0	0
	10	50	14	71.4	7.1	10
20	1	5	13	100.0	38.5	38
	5	25	15	93.3	26.7	29
	10	50	13	92.3	30.8	33
25	1	5	14	85.7	35.0	41
	5	25	16	96.2	50.0	52
	10	50	15	96.7	53.3	55
30	1	5	12	100.0	41.8	42
	5	25	12	91.6	75.0	81
	10	50	13	100.0	76.9	77

* In no case did leg regenerates younger than 30 days *in vivo* produce cuticle or setae unless treated with molting hormone *in vitro*.

leg regenerates older than 20 days. The mean frequency of seta formation increased fourfold between 15 and 20 days and doubled again between 20 and 30 days, as did the seta/cuticle ratio. These differences were significant at the 95% level of confidence. Within the 30-day age group, there was also a significant difference between the 1 and 10 μg/ml dose levels.

The effects of a wider range of doses on 25-day-old leg regenerates are shown in Figure 7. From these results, we can see that when the slopes for seta formation and cuticle deposition are extrapolated, they have a common origin at the 2% level of response (0.35 μgd), but that the rate of increase in response at a given dose is fourfold greater for cuticle deposition than for seta formation. The frequency of cuticle deposition increased with an increase in dose up to 2 μgd. Above

this dose, 99% responses occasionally occurred, but it happened more frequently that one or two specimens failed to respond at all, even at high levels. Since this was apparently a random occurrence, we used the mean response for doses above 3 μgd as the practical maximum response. The response of seta formation rose more slowly than that of cuticle deposition, but even at the maximum response level of 18 μgd, only 50% of the specimens produced setae.

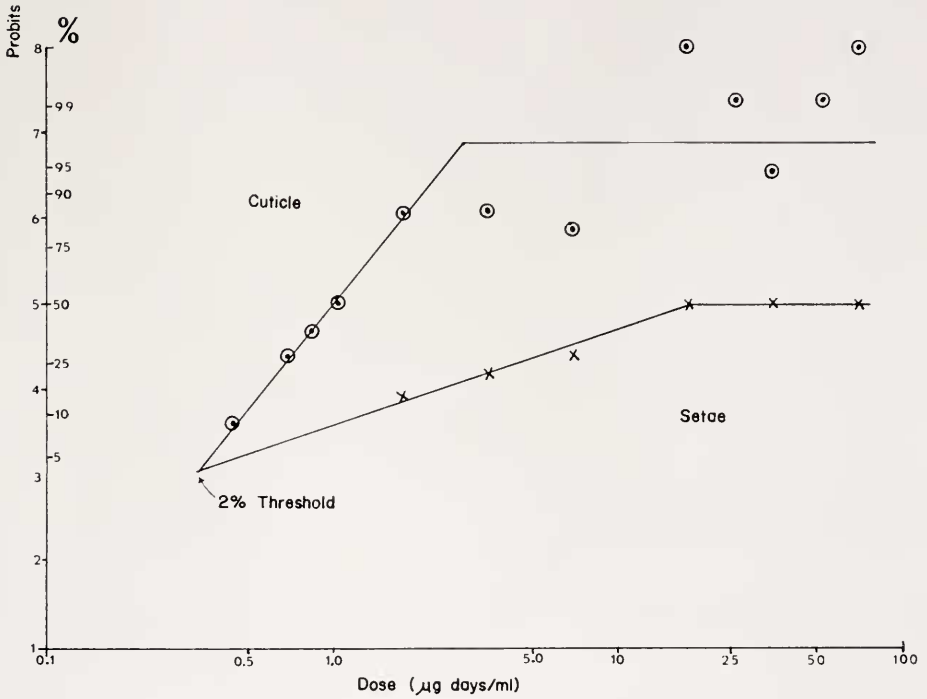


FIGURE 7. Effect of β-ecdysone on cuticle deposition and seta formation in 25-day-old cockroach leg regenerates; regression for cuticle deposition (⊙) up to 5 days is $y = 0.89 + 4.10$ (10×); $r = 0.99$ and regression for seta formation (×) up to 15 days is $y = 2.56 + 1.06$ (10× ; $r = 0.99$.

It was apparent from our earlier work that the age of the specimen *in vivo* was also a critical factor in determining the response (Marks and Leopold, 1971). Therefore, we ran an additional series of experiments in which the dose was held constant and the age of the specimen *in vivo* was varied from 10 to 30 days. The dose of hormone was kept constant at 25 μgd, and the frequencies of cuticle deposition and seta formation were plotted against the age of the leg regenerates *in vivo*. Under these conditions, the frequency of cuticle deposition rose rapidly from 10 to 25 days. However, the frequency of seta formation rose only after a delay of 15 days (Fig. 8). In both cases, the coefficient of correlation was sufficiently high (better than 0.90) to permit extrapolation of the lines to the 99.9% response level. When this was done, the two slopes met at a point corresponding to an age of 55 days *in vivo*. These data fit in well with those from our earlier study in which

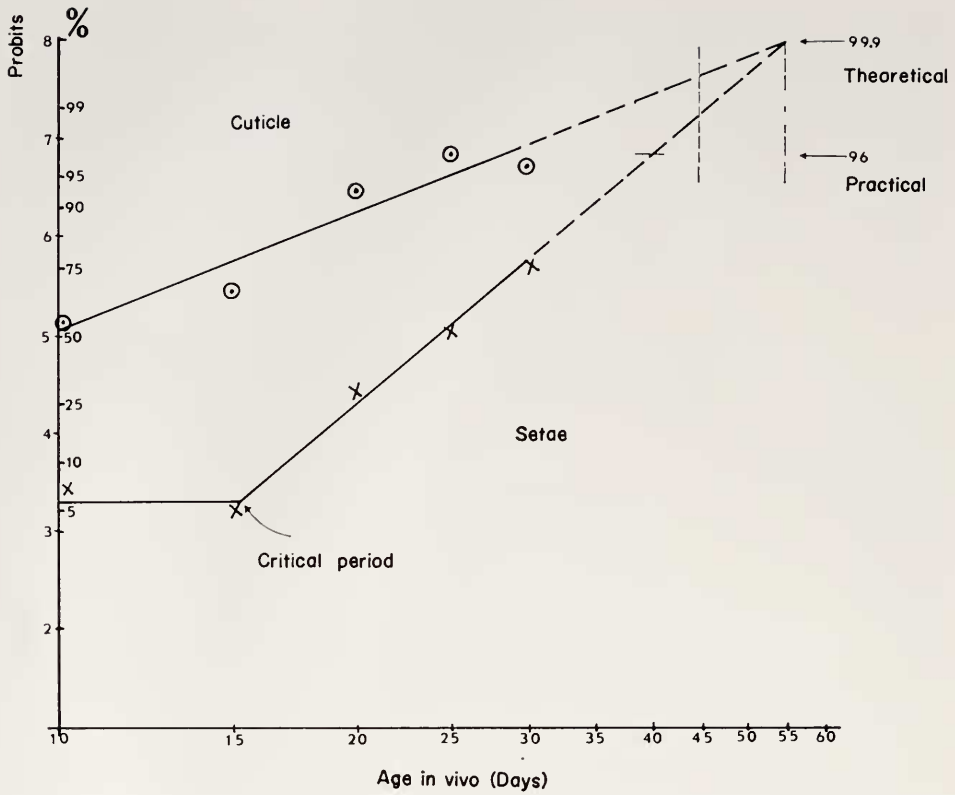


FIGURE 8. Effect of age in vivo on the induction by β-ecdysone (25 μgd) of cuticle deposition and seta formation in cockroach leg regenerates; regression for cuticle deposition (⊙) is $y = 1.13 + 3.92x$; $r = 0.92$ and regression for seta formation (×) from 15 to 30 days is $y = 8.15x - 6.31$; $r = 0.99$.

we found that 75% of 30-day-old regenerates that were induced to molt also bore setae; that 54% of 35-day-old regenerates developed without exogenous hormone, and that by 45 days, the new cuticle with setae was present at the time of explantation (Marks and Leopold, 1971). The average stadium was 52 days with a range of 45–60. Thus the slopes for cuticle deposition and seta formation met at an

TABLE II

The effect of puromycin on the induction by β-ecdysone of cuticle deposition and seta formation in vitro

Dose (μg/ml)		Days *in vitro*	Number specimens	Percentage response	
β-ecdysone	Puromycin			Cuticle	Seta
None	None	3	15	0	0
2.5	None	3	15	96	33
2.5	0.5	3	15	93	0

age well within the normal range for molting and demonstrated graphically the coordination between the processes of morphogenesis and cuticle formation.

When puromycin was added to cultured 25-day leg regenerates along with 2.5 μg/ml of β-ecdysone, cuticle was produced by more than 90% of the specimens, but no seta formation occurred. In comparison, 33% of the control specimens produced setae. Thus, a dose of puromycin sufficient to halt seta formation entirely had no apparent effect on the frequency of cuticle deposition (Table II).

Discussion

All cuticular structures formed on the cultured leg regenerates are the results of morphogenic processes, but only setae could be readily identified as unmistakable cases of differentiation since it was apparent from our time-lapse photographs of seta formation *in vitro* that the tormogen and trichogen cells arise directly from cuboidal epidermal cells. This occurred several hours before the first evidences of cuticle deposition appeared (Marks, 1972b).

It was also apparent from our time-dose studies that these processes depend on the age of the tissue *in vivo* and on the dose of β-ecdysone received. If we use the frequency of response to a given level of hormone as a measure of the readiness of the tissue to respond, then the frequency of cuticle deposition represents molt readiness and that of seta formation represents differentiation readiness. However, the degree of readiness or competence to respond to doses of exogenous hormone depends on the age of the tissue *in vivo*.

Ohtaki *et al.* (1968), in their work with larvae of *Sarcophaga,* found that at no time during the last larval stadium did the titer of ecdysone reach the level required to induce molting, but at no time was it entirely absent. Nevertheless, when the larvae reached a given age, pupation took place. They explained this by postulating that the gradual accumulation of covert "hormone-initiated events" (hie) over a period of time eventually reached a number sufficient to trigger pupation. Marks (1972a) reported a similar situation with cockroach leg regenerates in which repetition of subthreshold doses of β-ecdysone *in vitro* eventually triggered cuticle deposition. If we assume that hie accumulate throughout the stadium in response to a low but constant titer of hormone *in vivo,* then the competence of tissue to respond reflects the number of hie present at any given time.

In the present study, when mature leg regenerates were treated with β-ecdysone *in vitro,* the number of hie accumulated *in vivo* was augmented artificially, and cuticle deposition and the differentiation of setae occurred within a few days. However, these two processes—molting and differentiation—responded differently to the augmented accumulation of hie. As the hormone dosage *in vitro* was increased, the frequency of cuticle deposition in 25-day-old regenerates increased rapidly to 96%, but seta formation rose only to an age-limited maximum. When the dose was held constant and the age *in vivo* was increased, the age-limited maximum increased progressively. Apparently, the initiation of cuticle deposition depends almost entirely on the accumulation of hie, but seta formation involves an additional age-dependent factor. The existence of this additional factor was demonstrated by the addition of puromycin, which effectively blocked seta formation but did not affect cuticle deposition. Although seta formation is dependent on the same β-ecdysone trigger, it also requires simultaneous protein synthesis.

The changes in the relative sensitivities of seta formation and cuticle deposition to a given number of hie provide the coordination that assures simultaneous completion of these two processes. Thus, during the first 15 days *in vitro*, the leg regenerates exhibited a high degree of molt readiness but a low degree of differentiation readiness, and the induction of molting by the addition of exogenous hormone during this critical period resulted in cuticle deposition without differentiation. A comparable situation *in vivo* resulted in the formation of a sclerotized papilla when a leg was removed late in the stadium (see O'Farrell and Stock, 1953).

In his studies of diapausing pupae of *Samia cynthia* (Drury), Williams (1968) found that the administration of large doses of β-ecdysone and other compounds with molting hormone activity before the onset of adult development caused an acceleration of the events related to metamorphosis. The doses also caused numerous developmental abnormalities that included underdeveloped legs and genitalia, patches of pupal cuticle, and the loss of normal cuticular ornamentation. Such abnormalities probably occurred because of the partial failure of the differentiation processes that normally accompany adult development. When such doses were given 60 hr after the onset of adult development, the resulting moths were normal. Socha and Senhal (1972) obtained similar results in their experiments with *Tenebrio*. Also, Judy and Gilbert (1970) reported that treatment of hindgut from pupae of *Manduca* sexta (L.) with β-ecdysone early in the instar caused cuticle to be deposited on the rectal pads before development was complete. Apparently, the premature deposition of cuticle with accompanying incomplete differentiation of the epidermal cells is a common response of tissues when molting hormone is applied early in the development cycle.

When the results of the present experiments *in vitro* are compared with the *in vivo* experiments of Williams (1968) a general agreement is apparent. The speeding up of developmental processes in response to exogenous hormone applied late in the cycle and the loss of setae on the cuticle in response to exogenous hormone applied early in the cycle are common to both experiments. The suggestion that cuticle deposition stops further differentiation of epidermal structures is supported by both the time-dose and time-lapse studies.

The present study thus confirms the hypothesis of Williams (1968) and extends it to include regeneration in paurometabolous insects. It also provides some insight into the way in which the processes leading to cuticle deposition interact with the processes leading to tissue competence. In addition, our results suggest that the use of *in vitro* techniques may make it possible to separate the process of seta formation into its various component parts. These can then be studied in isolation. Such studies may eventually lead us to a better understanding of the nature of tissue competence.

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Summary

A study of the effects of β-ecdysone on the initiation of cuticle deposition and seta formation by cockroach leg regenerates *in vitro* showed that both processes are

ecdysone-dependent and are initiated by the same threshold dose, but the responses differ qualitatively and quantitatively. The initiation of cuticle deposition depends primarily on the accumulation of hormone-initiated events by the target tissue. The initiation of seta formation has an additional requirement for simultaneous protein synthesis.

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